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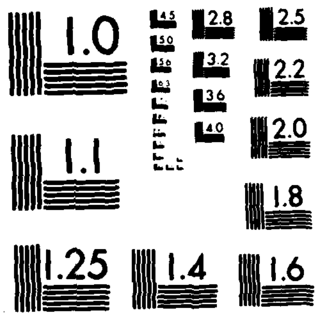

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RAPID DIAGNOSIS OF ARBOVIRUS AND ARENAVIRUS INFECTIONS BY IMMUNOFLUORESCENCE

FOURTH ANNUAL REPORT

Jordi Casals-Ariet, M.D.

January 1981

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Yale University School of Medicine
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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

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Inactivation of yellow fever virus-carrying spot-slides can be accomplished by immersion in dilute betapropiolactone however, the quality of the slides may be impaired by this procedure.

The immunofluorescence test with spot-slides prepared with viral isolates submitted for identification has continued to prove a time and labor saving procedure for antigenic grouping of strains; the method has been used with strains origination in Fiji, Uganda and South Africa.

Small amounts of Congo-Crimean hemorrhagic fever virus in the form of dilutions of a stock suspension, have been detected in 24 hours by observation with the immunofluorescence test of cell monolayers in chamber-slides at daily intervals following inoculation.

Seroepidemiological surveys with spot-slides and immunofluorescence test have demonstrated the presence of antibodies against Ebola virus in Ethiopia, Ghana, Senegal, Sudan and Cameroon. The antibodies in Ethiopia were found in individuals bled 13 or 14 years before the first reported outbreaks of Ebola hemorrhagic fever.

SUMMARY

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FOREWORD

In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care", as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences - National Research Council.

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BODY OF REPORT

I. Statement of problem

Human diseases caused by certain arboviruses, arenaviruses and special viral pathogens as yet unclassified, constitute a particular threat to the group or community in that the viruses in question have a tendency, given favorable circumstances, to infect by contagion; furthermore, the diseases can be severe, with high mortality and if a specific treatment such as serum therapy is entertained, it is more effective if given early. The problem under consideration is, therefore, how to diagnose specifically the disease at the earliest possible moment.

At the individual level the fastest way to a diagnosis is by visualization and specific identification of the virus or its antigens directly in clinical specimens. If this approach fails and an amplifying system, animal or cell culture, is used, it may be possible to shorten the required time by detection of the antigen in the amplifying system before disease or lesions are apparent. Failing to isolate a virus, detection of early developing antibodies, IgM, can give a relatively early answer.

Information on geographic distribution of viral pathogens is a considerable help towards rapid diagnosis of the disease that they cause, by directing attention to the pathogens known to be present in a given area. This particular aspect of the problem of rapid diagnosis, namely, geographic distribution of the viruses under consideration, has taken an important part of the effort during the period under review. For reasons to be given later, the problem has been attacked by determination of the presence of antibodies in man by means of the indirect immunofluorescence (IF) test.

II. Background

Beginning with 1967 several new, or newly recognized, viral hemorrhagic fevers of man appeared out of Africa which for various reasons have caused considerable concern: Marburg disease in 1967 (Martini, 1971), Lassa fever in 1969 (Frame _et al_., 1970) and Ebola hemorrhagic fever in 1976 (Breman _et al_., 1978; Bowen _et al_., 1978). In addition, other viral hemorrhagic fevers known from earlier periods, Congo-Crimean hemorrhagic fever (CCHF) in Eurasia and Africa and hemorrhagic fever with renal syndrome (HFRS) or Korean hemorrhagic fever (KHF) in Eurasia, have lately commanded increased attention in that they have appeared in areas heretofore not known to be endemic and have caused substantial morbidity, the former in Iraq and Dubai in 1979 (Al-Tikriti _et al._, 1981; Anonymous, 1979), the latter in central and southern provinces of China (Lee _et al_., 1980) and in Japan in 1960 and later (Anonymous, 1980). To these so-called "special pathogens" can be added Rift Valley fever (RVF) virus which although known to cause disease, even fatal, in man, was not considered dangerous but rather causing a mild dengue-like disease, until 1977 when a large epidemic with severe complications and high mortality appeared in a new area, Egypt (El-Akkad, 1979).

Rapid diagnosis of these diseases has been attempted by detection of virus directly in clinical specimens; IF has been applied to early diagnosis of Lassa

fever through detection of antigen in conjunctival smears (Anonymous, 1980); electronmicroscopy (EM) has permitted visualization of Ebola and Marburg viruses in the blood of patients (Bowen et al., 1978; Peters and Muller, 1968); and the viremia titers in RVF is so high that it has been suggested that the acute serum could be used as a source of hemagglutinating antigen (Anonymous, 1980).

Visualization of arbovirus and arenavirus virions in clinical specimens, however, is accomplished rarely; it is generally necessary to use an amplifying host, animal or cell culture. The antigen is identified, usually by IF, with inoculated animals or cells by sampling before disease, death or CPE. This procedure has been applied to the diagnosis of dengue by inoculation of mosquitoes (Kuberski and Rosen, 1977) or tissue cultures (Digoutte et al., 1979; Tesh, 1979); and of St. Louis encephalitis virus in tissue cultures (Mattos et al, 1979).

Serological tests as an aid to diagnosis of current disease caused by arboviruses, arenaviruses and special pathogens is not a rapid method as it is based on the demonstration of antibody development between early and later sera. A valuable lead with a single serum sample is given by a high antibody titer; or by detection of IgM antibodies.

While with arboviruses, particularly togaviridae, the hemagglutination-inhibition (HI) and neutralization tests can give positive results at a relatively early period after onset, these tests have limited application to early diagnosis of arenavirus and special pathogen diseases. No HI is applicable, except to RVF and only to some strains of CCHF virus (Casals and Tignor, 1974). The neutralization test has not been much used in connection with diagnosis of current disease caused by arenaviruses, Ebola and Marburg; antibodies are late in appearing, probably several months in Lassa fever (Anonymous, 1980) and the test is not a simple procedure with LCM (Hotchin and Kinch, 1975); attempts to show neutralization of Ebola virus have not been successful (Webb et al., 1978). Furthermore, even moderate scale use of the neutralization test with class 4 pathogens is not practical; and its use in the absence of proper containment facilities is not acceptable by current regulations.

Since 1975 the IF test has become the standard method for serological diagnosis of arenavirus diseases and of diseases caused by the Marburg type pathogens, supplanting the complement-fixation (CF) test which is less sensitive; the IF test has also been used with arboviruses. The IF test has been applied to the diagnosis of Colorado tick fever (Emmons et al., 1969); Bolivian and Argentinian hemorrhagic fevers (Peters et al., 1973; Grela et al., 1975); Lassa fever (Wulff and Lange, 1975); CCHF (Zgurskaya et al., 1975; Burney et al., 1978); and Marburg and Ebola fevers (Webb et al., 1978; Bowen et al., 1978).

Other serological techniques may be considered for early diagnosis or seroepidemiological surveys with the viruses under consideration, but their application has thus far been limited. The indirect hemagglutination test and its inhibition has been reported for Tacaribe and LCM viruses (Gajdamovich et al., 1975); the agar gel diffusion and precipitation test has been used by Soviet investigators with CCHF (Chumakov and Smirnova, 1972; Karinskaya et al., 1974); the solid phase radioimmunoassay using protein-A-bearing Staphylococcus aureus as the solid phase immunoadsorbent has been successfully applied to arboviruses (Jahrling et al., 1978); and the enzyme-linked immunosorbent assay (ELISA) has been recently

reported with arboviruses (Frazier and Shope, 1979).

As indicated in section I, seroepidemiological surveys will serve the purpose of mapping out the distribution of the viruses under consideration. The IF test employing inactivated spot-slides is currently the most practical and safe tet available; it has already been applied with excellent results to some agents such as Lassa virus (Frame et al., 1980; Bloch, 1979), Ebola and Marburg (Johnson, personal communication, 1980) and KHF (Lee et al., 1980).

III. Approach

The working hypothesis made at the outset of this project was that an early diagnosis of infection by arboviruses and arenaviruses could be made in a simple and practical manner by means of the IF test. The hypothesis was to be tested by applying the tet to the detection of antigen, virological diagnosis, and of antibody, serological diagnosis.

As it was conjectured that virus-containing specimens from current cases would be hard to come by, the feasibility of rapid recognition of antigen was to be tested either by producing an infection in small laboratory animals and testing their early sera for viremia; or by using viral stocks as a substitute for clinical specimens as a source of pathogens.

The realization that early diagnosis of certain arbovirus diseases -- CCHF, RVF -- and of diseases caused by viruses designated as "special pathogens" --Lassa, Marburg, Ebola, KHF -- could be improved by previous knowledge of the world distribution of these agents, stimulated an additional phase of research, seroepidemiological surveys. The surveys were initiated and are being continued, using as antigens in the IF test cells from infected tissue cultures deposited on microscope slides, fixed with acetone (spot-slides) and stored at low temperature.

IV. Materials and Methods

Viruses. The viruses and strains used were: Banzi, SA H336; Congo-Crimean hemorrhagic fever, IbAr10200; Japanese encephalitis, Nakayama; Langat, TP21; Pongola, MP 781; Rocio, San Paulo ; Sicilian phlebotomus fever, Sabin; West Nile, Eg101; yellow fever, Asibi. the viruses are maintained as 10% infected newborn mouse brain tissue suspensions held lyophilized or wet frozen at -60°C.

Immune reagents. Reference reagents available at the World Reference Center, YARU, were used, including polyvalent grouping and monovalent reagents. These reagents were mouse sera and ascitic fluids and sera from hamster, guinea pig and rabbit, kept wet frozen at -20°C or lyophilized at 4°C.

Cell cultures. The following cells routinely maintained in this laboratory have been used: VERO, BHK-21, LLC-MK2 and CER. The cells were maintained in 150 cm^2 plastic bottles; transfers were made every 8-10 days for VERO and LLC-MK2, every 4-6 days for the other cells. Fresh cultures were started from cells held in liquid nitrogen when it seemed warranted.

Animals. The following have been used: mouse (*Mus musculus*), Swiss outbred;

hamster (Mesocricetus auratus); guinea pig (Cavia porcellus); and rabbit (Oryctolagus cuniculus). The animals were purchased from commercial dealers; mice were used as newborn, 1 to 5 days old, or as adults, as required.

Bleeding of all animals and intracerebral inoculations were done under deep ether anesthesia. Whole bloods or sera to be tested for infective virus were stored at -60°C; sera for antibody tests, at -20°C.

Preparation of spot-slides. In essence the method used, described in full detail before in the Second Annual Report, January 1979, was as follows.

Cell cultures in 150 cm^2 plastic bottles showing a complete monolayer were infected with a virus at an MOI between 1 and 100. When CPE ws 1+ or 2+, or after a predetermined number of days with viruses that replicate with no CPE, the cells were harvested, dispersed with trypsin and versene (no versene was used with BHK-21 and CER cells), washed with phosphate buffered saline (PBS) and generally adjusted to a 3×10^6 cells/ml count; similar suspensions were prepared with uninfected cells. The two suspensions were mixed in a proportion of 3 or 2 parts infected to 1 of uninfected cells; the cell suspension mixture in volumes of 0.01ml (or 3×10^4 cells) was dropped on the surface of teflon-coated slides (Cel Line Associates, Minotola, N.J.) which have 12 circular areas 5 mm in diameter. The drops were allowed to dry by holding the slides at 37°C for 30 minutes; the slides were next immersed in acetone for 10-12 minutes and stored at -60°C. Slides have been used nearly 2 years after preparation with excellent results.

For polyvalent slides, individual suspensions were prepared as described above with each of the viruses; a mixture of equal parts of each infected suspension and of a non-infected suspension of one of the cell lines was made and used.

Preparation of chamber slides. Tissue culture slides 75 x 25 mm with 4 chambers were used (Lab-Tek Products, Miles Laboratories, Neperville, Illinois). Monolayers under fluid medium were prepared by seeding each chamber with 2 to 2.5×10^5 cells in a volume of 0.8 or 0.9 ml of growth medium; the slides were held in a CO_2 incubator. One to three days later the monolayers were infected with the virus, or with a virus-serum mixture for a neutralizaiton test, in a 0.1 ml volume; maintenance medium was added after a period of adsorption of 1h and the sides again held at 37°C in the CO_2 incubator. At the desired time, 1 to 5 or 6 days later, the chambers were drained and removed and the slides fixed in acetone for IF staining.

Immunofluorescence test. Isothyocyanate conjugates were purchased from commercial dealers; preparations in routine use were conjugated anti-globulins for man, mouse, hamster, guinea pig and rabbit. The indirect technique used was a standard laboratory procedure (Gardner and McQuillin, 1975); Evans' blue at a final dilution of 1:10,000 was used as a counter-stain. An Olympus "Vanox" incident light microscope set for blue fluorescence and a mercury burner light source were used. When viewed with x40 and x100 immersion objectives, glycerol directly over the slide without a cover-slip was used.

For routine testing, human sera were screened at dilution 1:4; positive sera were titrated at increasing two-fold dilutions. On accasion sera were tested undiluted and at dilution 1:2; in these instances and also when it ws deemed

necessary to ascertain whether weak reactions were truly specific IF or the result of adsorption of dye by precipitates in the serum, the sera were extracted with acetone and ethyl ether (Casals and Tignor, 1974).

The results of the IF tests are given in this report according to the following rating. Sera tested only at dilution 1:4 are rated "strongly positive" when the brightness or intensity of fluorescence was 3+ or 4+, in a scale from 0 to 4+; "medium positive", when it was 2+; and "weak positive", when it was 1+. Sera positive at dilution 1:8 or higher are all rated "strongly positive".

Other serological tests. Other tests used, CF, HI and neutralization tests in mice or cell cultures have been described in detail (Casals, 1967; Schmidt, 1979). The CF and HI tests wre semi-micromethods with a total volume of reagents of 0.15 ml (6 drops) and 0.1 ml (4 drops) respectively.

Human sera. As far as could be ascertained all sera for serological surveys were obtained from seemingly well individuals. The sera were stored wet frozen at -20°C, with the exception of the sera from Ethiopia which had been lyophilized at the Pasteur Institute, Addis Ababa, at the time of collection and subsequently stored at -20°C.

V. Results

Polyvalent spot-slides for flaviviruses (Group B). A set of slides was prepared with a mixture of cells infected with the following viruses: Banzi, in BHK-21; Japanese encephalitis, in VERO; Langat, in BHK-21; Rocio, in VERO; and yellow fever, in VERO. Infected cells were mixed in equal proportions; the number of cells in the mixture was 4×10^6 cells/ml, therefore the number of cells in each spot was 4×10^4 cells. Mouse hyperimmune sera for flaviviruses were tested in dilutions 1:8 and 1:16 with the slides, by IF test, with the result shown in Table 1. Most sera gave positive reaction at dilution 1:16, except Central European tick-borne encephalitis (CETBE) and Kokobera; although it was subsequently determined that the CETBE serum had no homologous antibodies, it still remains that not all the group B antisera tested gave positive reactions with this set of slides. In this respect, these polyvalent slides resembled a previously prepared set (see 3rd annual report, January 1980) which also failed to detect antibodies in some of the group B antisera used. It seems unavoidable that, in order to have maximum flaviviruses coverage, 2 subsets of group B polyvalent slides will have to be prepared, one for the tick-borne viruses, another for the other agents.

Inactivation of spot-slides. CCHF virus spot-slides that had been stored at -60°C for 90 days were irradiated with ultraviolet light (UVL) from the burner in a Baker Biogard hood, the distance between burner and slides being 45 cm; a set of slides was irradiated for 10 minutes, another for 20 minutes and a third was not irradiated. Irradiated and non-irradiated slides were tested for virus; drops of diluent were deposited on the spots and the tissue scraped off the glass. The total volume of diluent used for each slide (10 spots) was 0.5 ml; this suspension was called "undiluted". The 3 suspensions, 10 and 20 minutes irradiated and and non-irradiated, were diluted to 10^{-3} and intracerebrally inoculated to 3-day-old mice in groups of 8 mice per dilution. No mice died or showed signs of illness, including those inoculated with non-irradiated slides; in this respect the experiment failed to show whether UVL had inactivated the virus. On the other

hand, the experiment showed that the method used for preparation of slides -- heating at 37°C for 30 minutes and immersion in acetone for 10-12 minutes -- followed by 3 months storage at 60°C resulted in virus inactivation. No difference was observed between irradiated and non-irradiated slides when an immune mouse serum was titrated with them; the intensity of fluorescence, location of fluorescent antigen in the cells and titer of the serum were the same in the 3 sets.

Yellow fever, Asibi strain, spot-slides were inactivated by immersion in betapropiolactone (BPL) at dilution 0.1% in PBS, pH 7.2; following this treatment, the slides were dried and stored at -60°C until tested for virus and in the IF test. Slides, treated with BPL and untreated, were tested for virulence as described in the preceding paragraph and for efficacy in the IF test by titration of an immune mouse serum. Two attempts were carried out with slides that had been stored 4 and 5 months, respectively, at -60°C after preparation; the result of the intracerebral titrations in newborn mice is shown in Table 2.

Following 10 minutes inactivation in experiment #1 there was possibly some residual virus; in test #2, the deaths are considered non-specific as they occurred 10-12 days after mouse inoculation. In the IF test, the slides in experiment #1 after 10 minutes exposure to BPL, were nearly as good as the untreated ones. In experiment #2 all treated slides regardless of length of treatment were alike and definitely inferior to the controls. The IF seemed bleached out and hard to read at x20, somewhat better at x 40 magnification; however, the titer of the serum was the same against the 3 sets of slides. More work is needed in order to determine whether UVL or BPL are effective means to inactivate spot-slides while preserving their efficacy.

Application of IF to virus identification. During the current period the IF test was employed on several occasions as a practical rapid step for identification of viral isolates. A strain from Fiji, #41451, from a febrile patient, was inoculated to BHK-21, VERO, CER, Aedes albopictus and A. pseudoscutellaris cell cultures; spot-slides were prepared with the cells and tested against grouping polyvalent mouse immune ascitic fluids for groups A, B and an additional 10, with the result that only group A fluid gave a positive reaction with the slides. The isolate was ultimately identified as Ross River virus.

A similar procedure was followed with 2 strains isolated in Uganda, Ug MP-15332 and UgZ-52969 and one in South Africa, SA An-24630. Cell cultures BHK-21, VERO and CER were inoculated with the strains, spot-slides prepared and tested with polyvalent grouping reagents. Isolate SA An - 24630 reacted only with the group B fluid; Ug MP - 15332 only with Bwamba; and UgZ - 52969 with none of 28 grouping fluids. In the course of time, SA An - 24630 was identified as a strain of West Nile, Ug MP - 15332 as Pongola and Ug Z - 52969 as a new virus distantly related to Yogue virus.

In a converse procedure, an immune mouse serum prepared against a strain, CS-122, submitted from Australia, was tested with polyvalent groups A and B slides; the serum reacted only with the latter. The virus was finally identified as a new group B agent.

Application of IF to rapid identification of CCHF virus. In the Annual Report for the 3rd period, January 1980, it was shown that rapid identification of JE, Banzi and Junin viruses could be done by daily processing for IF of cell monolayers

in chamber-slides inoculated with the viruses.

A similar study was carried out with CCHF virus. A virus stock, strain Ib Ar 10200, having an LD_{50} of $10^{-6.5}$/0.02 ml by intracerebral inoculation to 2-day-old mice was used in dilutions 10^{-3}, 10^{-4}, and 10^{-5}. Monolayers of CER cells were prepared in 4-chambered slides by seeding 2 x 10^5 cells in 0.9 ml of medium in each chamber; 24 hours later, 4 slides were inoculated with 0.1 ml of each virus dilution, the 4th chamber receiving diluent instead of virus. No CPE was noted during the duration of the experiment. A slide was examined in the IF test on days 1, 2, 3 and 5 after inoculation of the virus; as source of antibody was used an anti-CCHF serum, having a titer of 1:512, at dilution 1:10. The result of the tests is shown in Table 3.

As shown in Table 3, the number of $ICLD_{50}$ doses contained in 0.1 ml of each dilution was, respectively, 1.5 x 10^4, 1.5 X 10^3 and 1.5 X 10^2. The IF reaction was strongly positive with all the combinations of time and dilutions, with a 4+ or maximum fluorescence in all instances except a 3+ with the smallest inoculum (10^{-5}) containing only 300 LD_{50}, tested 1 day after infection.

Application of IF to diagnosis of Korean hemorrhagic fever. Diagnosis of KHF has been made possible by the propagation of the etiological agent in cell cultures and its detection by IF (Lee *et al*., 1978; Lee *et al*., 1980). Spot-slides bearing suspensions of cells of human carcinoma A-549, infected with KHF were supplied by Dr. G. French, USAMRIID; control, uninfected slides were also supplied. Three sets of available sera were tested in an attempt to determine the suitability of these slides as diagnostic antigens: a) Fifteen convalescent sera from persons admitted to the Hospital of the Hubei Provincial Medical College, Wuchang, China, between 1975 and 1979 with the clinical diagnosis of epidemic hemorrhagic fever (Cohen *et al.*, in preparation, 1981); the sera were supplied by Professor C.M. Hsiang, of that Medical School. b) Ten paired sera from patients admitted to 3 hospitals in South Korea in 1974, clinically diagnosed as KHF; the sera were supplied by Dr. K. H. Kim, National Institute of Health, Seoul, Korea. c) Single sera taken during convalescence from 10 U.S. Army personnel, with a clinical diagnosis of KHF in 1967-68; the sera were given by Dr. Ned H. Wiebenga, Chief, Epidemiology Branch, Department of Health, Hawaii.

The sera were tested by IF at dilution 1:4 only, with the exception of two that were titrated in increasing 4-fold dilutions. All 15 sera in group a) gave positive reactions with readings of 3+ or 4+, except one serum that gave a questionable reaction; the reactions with the control slides was uniformly negative with these as well as with the remaining sera. The results with the sera in groups b) and c) are given in Table 4. Intense positive reactions were given by all patients except KHF-5, which may have been a false clinical diagnosis. Acute sera taken as early as 6 days from onset were strongly positive; titrations of two sera, J.H.L. and J.D.Y., resulted in titers of 1:2048 and 1:512, respectively.

Antibodies against yellow fever virus after vaccination with 17D: comparison of neutralization, HI, IF and ELISA tests. Antigen for ELISA test was prepared by precipitation of the virus grown in cell culture by polyethylene glycol and centrifugation in a discontinuous sucrose gradient. The result of the test was recorded with an ELISA-reader (Titertek) and the titer of a serum was determined by comparing its regression analysis slope with that of a control serum. Pre- and

post-vaccination sera from 13 individuals were compared by plaque reduction neutralization test at 90% and 50% reduction, HI, IF and ELISA, with the results shown in Table 5. The conclusion of this investigation was that following vaccination with 17-D yellow fever vaccine, neither ELISA nor IF tests detect antibodies, with few low titer exceptions, in persons who were positive both by plaque reduction and HI tests. Two of the 13 individuals who were strongly positive by ELISA after vaccination were also positive with the same titers before vaccination; the reaction was non-specific, as the plaque reduction and HI tests show.

Serological surveys by IF test. Human sera from Ghana, Ethiopia, Sudan, Senegal, Cameroon, Liberia, Greece, Pakistan and United States were variously tested for antibodies against Ebola, Lassa, Marburg, KHF and CCHF viruses. The results are summarized in Table 6.

Ghana. Over 1200 sera were collected by Dr. N.S. Quao during the summer of 1975, of which 344 have been tested during the current period. The sera tested derived from residents of the Brong-Ahafo region; they were tested against polyvalent Ebola, Lassa and Marburg (ELM) slides supplied by Dr. K.M. Johnson, CDC, Atlanta, Ga. and 31 that were positive were subsequently tested with monovalent Ebola, Lassa and Marburg slides. In addition, 47 sera were tested for antibodies against CCHF virus. As shown in Table 6, 20 sera were strongly positive under the criteria adopted and 17 were medium positive. The titers of 7 sera thus far titrated were; 1:32, 3 sera; 1:16, 3 sera; and 1:8, one serum. Tests with monovalent slides were positive only for Ebola; 5 sera that had been positive with polyvalent ELM sides, failed to react positively with any of the monovalent slides. It is conceivable that the individual antigens used in the polyvalent slides were more potent than those used for the monovalent slides. It should be pointed out that those sera were collected about one year before the disease, Ebola hemorrhagic fever, was observed for the first recorded time.

Ethiopia. In 1962-63 a large number of sera were collected in that country by Dr. C. Serie, at the time director of the Pasteur Institute, Addis Ababa, of which around 400 were submitted to the Rockefeller Foundation Virus Laboratory, the predecessor of YARU; thus far, 115 sera originating mainly in the valleys of the Awash and Blue Nile rivers have been tested against Ebola, Lassa and Marburg viruses. As shown in Table 6, 8 sera were strongly positive and 10 medium positive in the tests with ELM slides; only positive with Ebola virus were found with the monovalent slides. One serum gave a titer of 1:128, 2 sera titers of 1:32 or higher, and another 2 sera had titers of 1:16. These results show that antibodies capable of reacting with Ebola antigen were present in the population of Ethiopia 13 or 14 years before the disease was first recognized.

Sudan. A survey for antibodies against a large number of arboviruses and special pathogens is being conducted in this laboratory with sera collected in 1978-79 by Dr. J.M. Meegan, NAMRU-3, Cairo. The result of tests with ELM slides show that of 69 sera, 6 were strongly positive and 3 medium positive; while monovalent tests are still pending, it is provisionally assumed that the positive results are due to reaction with Ebola virus.

Senegal. About 350 sera were collected in February 1977 by Dr. W.G. Downs of these laboratories; 74 sera, originating mainly in the region of the lower Senegal

river, have been thus far tested, of which 7 were strongly positive and 1 medium positive with the ELM slides. Although no tests have as yet been carried out with monovalent slides, the aspect of the IF reaction is identical to that associated with Ebola rather than Lassa viruses.

Cameroon. Sera were collected in that country as part of a survey for antibodies against Lassa fever virus in equatorial and West Africa (see later, under Liberia). Forty-two sera originating in Garoua-Boulai have been tested with ELM and Lassa slides; positive reactions were observed only with ELM slides, 3 strong and 3 medium; a serum had a titer of 1:32, another a titer of 1:8. It is tentatively concluded that these antibodies are directed against Ebola rather than Marburg virus.

Liberia. A continuing large scale survey for anti-Lassa virus antibodies in several countries of equatorial and West Africa has been in operation for several years, in association with Dr. J.D. Frame, College of Physicians and Surgeons, Columbia University, New York. A total of 311 sera collected in 1978 and 1980 in Liberia, mainly in the areas of Foya and Zorzor, were tested this year against Lassa slides only; 32 sera were strongly positive and 19 medium positive, with 3 having titers in the range 1:256 and 1:512.

Greece. Sera were collected in northern Greece by Dr. A. Antoniadis, Aristotelian University of Thessaloniki, School of Medicine and tested by him at YARU. A total of 323 sera were tested by IF against CCHF virus and 29 against KHF virus; with the exception of 3 or 4 sera that gave questionably positive reactions against the former, all results were negative.

Pakistan. About 200 human sera collected in that country in 1978-79 were supplied by Dr. C. Hayes, Gorgas Memorial Laboratory, Panama; the sera are intended for a survey for CCHF and KHF virus antibodies. In the course of 1980, 49 sera were tested against CCHF and 24 against KHF, all with negative results.

United States. The fact that so many human sera from certain areas of Africa had antibodies that reacted with ELM slides in the absence of recognized disease caused by Ebola, Marburg and Lassa viruses, made it desirable to test sera from a contrasting population as a control for specificity of the reaction. To this end, sera from 35 residents in two areas of the United States, northeastern and Alabama, were included in tests with polyvalent ELM slides; all these sera were negative in blind tests.

<u>Spot-slides for IF tests supplied to USAMRIID</u>. As part of the contract, spot-slides were prepared, tested for specificity and adequacy and shipped to USAMRIID; two ampules of virus stocks and 6 x 0.5 ml of hyperimmune mouse serum or ascitic fluids were included in the shipments. The viruses and cells used and the number of slides supplied were:

Polyvalent group B, Banzi, Japanese encephalitis, Langat, Rocio and yellow fever; several cells; 200

Rocio, San Paulo, VERO; 200

Yellow fever, Asibi, VERO; 200

VI. Discussion

The need for rapid diagnosis of arbovirus and arenavirus dieases as well as of diseases caused by the Marburg-Ebola agents, is increasing as the occurrence of outbreaks of these diseases are more frequent. Under circumstances favoring the pathogen these diseases can be transmitted by contagion from person to person or from domestic livestock to man; they can appear in unsuspected and distant areas through airplane transportation of persons in the incubation or prodromal periods; and can endanger the lives of new arrivals in endemic areas. Furthermore, early diagnosis increases the probability of survival through administration of specific antisera when these are available and determines whether strict rules of isolation and managment are needed.

Identification of a virus directly in clinical specimens without using an amplifying system is, undoubtedly, the most rapid way to achieve a diagnosis. The method has not been universely successful, in great part because the virus concentration in blood or other materials or tissues may not be high enough; possibly, concentration of the pathogen by centrifugation and observation by electronmicroscopy may be useful. The possibility of preparing a hemagglutinating antigen (HA) using the acute serum of RVF patients has been mentioned; titers of 10^8 to 10^{10} LD_{50} per ml have been reported, which are sufficiently high to yield an acceptable HA antigen by acetone precipitation.

Our observation that as little as 300 LD_{50}per ml of CCHF virus could be specificaly detected 24 hours after inoculation of CER monolayers, is noteworthy. Studies by Butenko et al.(1974) have shown that viremia titers of the order of 10^5or 10^6per ml were not infrequent in patients with the disease within the first 10 days from onset; the amount of virus detected in our experiments was much below these values.

The reported results of seroepidemiological surveys by means of the IF test with spot-slides have shown that the technique is practical, safe and efective; while, in order to ascertain the complete specificity of the IF results, it woud be advisable to compare these results with those of other tets, the fact remains that no other serological tests are available with some of the viruses or are not dependable with others. Certain findings in the course of the surveys were notable, particularly that antibodies against Ebola virus were found in sera collected 13 or 14 years before the disease was discovered; whether these antibodies are due to infection with Ebola virus or with a related but distinct agent cannot now be established. Furthermore, antibodies against Ebola were found in Ethiopia and Ghana, countries which were not known heretofore to have the virus.

VII. Conclusions

1. Polyvalent spot-slides are useful for expediting the diagnosis of arbovirus groups A (alphavirus) and B (flavivirus) infections through detection of antibodies by immunofluorescence tests. Since the number of group A viruses of pathogenic importance for man is only 4 or 5, one set of slides should be sufficient for the group. However, it appears that in order to have nearly complete coverage in group B at least two subsets will be needed, preferably one for the tick-borne viruses and another for the mosquito-borne.

2. Identification of arbovirus isolates can be simplified to some extent through group placement of the isolate using the immunofluorescence test, spot-slides prepared with the problem isolate and polyvalent grouping immune reagents.

3. Based on observations with an experimental system, it appears that rapid specific diagnosis of Congo-Crimean hemorrhagic fever in man could be accomplished by immunofluorescence tests with monolayers in chamber-slides within 24 hours of reception of a virus containing acute serum.

4. Antibodies in man against Ebola virus, detected by immunofluorescence and spot-slides, have been found in areas of Africa in which the virus was not known to be present, Ghana and Ethiopia, and up to 14 years before the disease was first recognized. It is not possible to state at this time whether these are homologous antibodies or the result of exposure to a related, as yet undiscovered agent.

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Table 1

IF test with polyvalent group B spot-slides

Serum, mouse	Result 1:8	1:16
Banzi	3	4
CETBE	0	0
Dengue 1	2	±
Ilheus	4	4
JBE	4	4
Kokobera	trace	0
MVE	4	4
OHF	4	3
Powassan	4	3
SLE	2	trace
EEE	0	0

Intensity of IF in a scale 0 to 4

Table 2

Recovery of yellow fever virus from spot-slides before and after immersion in 0.1% betapropiolactone

Experiment	Treatment with BPL, minutes	Result of intracerebral inoculation to mice			
		UND	10^{-1}	10^{-2}	10^{-3}
#1	None	8/8	8/8	3/8	0/8
	10	2/8	0/8	0/8	0/8
#2	None	5/8	2/8	0/8	0/8
	10	*1/16	*2/16	0/16	
	20	0/16	0/16		
	30	0/16	0/16		

Mice dead of mice inoculated.
* possibly non-specific deaths.

Table 3

Rapid identification of CCHF virus by IF test of infected monolayers

Virus		Days after inoculation			
Dilution	$ICLD_{50}$	1	2	3	5
10^{-3}	$1.5x10^4$	4	4	4	4
10^{-4}	$1.5x10^3$	4	4	4	4
10^{-5}	$1.5x10^2$	3	4	4	4

Result: in a scale of 0 to 4.

Table 4

Diagnosis of Korean hemorrhagic fever: indirect immunofluorescence test

Patient identification	Serum Days from onset Acute	Serum Days from onset Late	Days from Hospital Admission	Result Acute	Result Late
KHF-1	8	15		3	4
KHF-2	6	13		3	4
KHF-3	6	16		3	2
KHF-4	7	21		4	3
KHF-5	6	35		0	0
KHF-6	13	35		3	3
KHF-7	12	34		3	4
KHF-8	7	38			4
KHF-9	11	42		4	4
KHF-10	14	45		4	4
D.L.			21		2
R.R.			28		4
J.H.L.			28		4(1:2048)
S.K.K.			32		4
J.D.V.			28		4(1:512)
M.C.			29		4
J.T.			30		4
L.G.			27		4
H.B.C.			26		3
J.K.L.			36		4

Sera tested at dilution 1:4; itensity of fluorescence in a 0 to 4 scale.

Table 5

Antibody development following vaccination of man with 17D yellow fever vaccine

Subject	Day bled after vaccination	Test, antigen, reciprocal of titer					
		PR		HI		ELISA	IF
		90%	50%	YF	JE	YF	YF
CR	-1	0	0	0	0	0	0
	76	16-32	32+	20	0	0	4?
JG	-1	0	0	0	0	0	0
	43	16-32	32+	40	0	0	0
GK	-7	0	0	0	0	0	0
	180	8-	32+	20	0	0	0
KO	-1	0	0	0	0	0	0
	270			40	0	0	0
SH	0			0	0	0	0
	15			40	0	80	0
BA	-6	0	0	0	0	320	0
	74	16	32+	40	0	320-640	0
	285			20	0	160-320	0
SB	0	0	0			0	
	75	8	32+	80+	10	0	4
MT	-2	0	0	0	0	0	0
	39	8-	16	40	0	0	0
JR	-1	0	0	0	0	320-640	0
	21	8-	32+	40	0	320	0
TT	0			0	0	0	0
	48			40	0	0	0
SH	-1	0	0	0	0	0	0
	790	8	32+	20	0	0	0
	870	8	32+	20	0	0	0
JK	?	0	8	20	0	0	4?
	?+28	16-32	32+	80+	0	0	4
MG	?	0	0	0	0	0	0
	?+197	32+	32+	80+	0	0	0

PR:plaque reduction; HI: hemagglutinatin-inhibition; IF: immunofluorescence; YF:yellow fever; JE: Japanese encephalitis.
First dilution of serum: PR, 1:8; HI, 1:10; ELISA, 1:20; IF, 1:4.

Table 6

Seroepidemiological surveys by indirect immunofluorescence (IF) test

Serum		ELM					Antigen and Result				CCHF	
Country	Year	Number tested	Positive Strong	Medium	Weak	Negative	Number tested	Number positive EBO	LAS	MAR	Number Tested	Positive
Ghana	1975	344	20	17	28	279	31*	26	0	0	47	0
Ethiopia	1962	115	8	10	7	90	12*	10	0	0		
Sudan	1979	69	6	3	5	55	4*	3	0	0		
Senegal	1977	74	7	1	6	60						
Cameroon	1980	42	3	3	2	34	42		0			

*Only sera positive with ELM antigen were tested. ELM, polyvalent Ebola-Lassa-Marburg antigen.

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